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ORIGINAL ARTICLE

Crx broadly modulates the pineal transcriptome

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Abstract

Cone-rod homeobox (*Crx*) encodes Crx, a transcription factor expressed selectively in retinal photoreceptors and pinealocytes, the major cell type of the pineal gland. In this study, the influence of Crx on the mammalian pineal gland was studied by light and electron microscopy and by use of microarray and qRTPCR technology, thereby extending previous studies on selected genes (Furukawa *et al.* 1999). Deletion of *Crx* was not found to alter pineal morphology, but was found to broadly modulate the mouse pineal transcriptome, characterized by a > 2-fold down-regulation of 543 genes and a > 2-fold up-regulation of 745 genes ($p < 0.05$). Of these, one of the most highly up-regulated (18-fold) was *Hoxc4*, a member of the Hox gene family, members of which are known to control gene expression cascades. During a 24-h period, a set of 51 genes exhibited differential day/night expression in pineal glands of wild-type animals; only eight of these were also day/night expressed in the $Crx^{-/-}$ pineal gland. However, in the $Crx^{-/-}$ pineal gland 41 genes exhibited differential night/day expression that was not seen in wild-type animals. These findings indicate that *Crx* broadly modulates the pineal transcriptome and also influences differential night/day gene expression in this tissue. Some effects of Crx deletion on the pineal transcriptome might be mediated by *Hoxc4* up-regulation.

Keywords: *Crx*, gene expression, *Hoxc4*, microarray, pineal gland, transcriptome profiling.

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Cone-rod homeobox (Crx) is a homeodomain transcription factor and member of the Otx family; Crx has been thought to play a critical role in determining and maintaining the phenotype of both pinealocytes and retinal photoreceptors (Chen *et al.* 1997; Furukawa *et al.* 1997; Rath *et al.* 2006, 2007). The selective expression of *Crx* in these cell types reflects their common evolutionary origin (Klein 2004; Mano and Fukada 2007). In the mammalian retina, Crx is essential for the normal development and maintenance of cones and rods (Furukawa *et al.* 1999) and regulates expression of the network of genes that characterize the retina (Hsiau *et al.* 2007). Elimination of *Crx* by disruption of the homeobox domain results in loss of the image-forming visual system (Furukawa *et al.* 1999) but not the non-image-forming visual system controlling circadian rhythms (Panda *et al.* 2003; Rovsing *et al.* 2010). $Crx^{-/-}$ mice exhibit suppressed circadian rhythms in locomotor activity, suggesting that Crx may directly or indirectly alter retinal ganglion cell output, the suprachiasmatic nucleus, other structures con-

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Abbreviations used: PCE, photoreceptor conserved element; ZT, zeitgeber time.

trolling locomotor activity, or a combination (Rovsing *et al.* 2010).

Whereas it is clear that the retina is severely impacted in the $Crx^{-/-}$ animal, the full impact of Crx deletion on the pineal gland is less clear. Studies on mouse pineal gland and on gene expression have indicated that Crx does not appear to alter pineal morphology, but does moderately modulate the abundance of *Aanat* transcripts (Li *et al.* 1998; Furukawa *et al.* 1999); *Aanat* is of special importance in the pineal gland because it encodes the enzyme that regulates the daily rhythm in melatonin production in vertebrates (Klein 2007). Studies in the chicken indicate that Crx is also involved in the control of expression of the last enzyme in melatonin synthesis, *Asmt/Hiomt* (acetylserotonin-*O*-methyltransferase/hydroxyindole-*O*-methyltransferase) (Bernard *et al.* 2001).

In the current report, we have pursued the goal of determining the full impact of Crx deletion on the pineal gland using anatomical methods and microarray technology, which has not been used previously to study the adult mouse pineal gland. Our results indicate that Crx plays a very broad role in modulating the pineal transcriptome and that a link exists between Crx and the homeobox gene *Hoxc4*.

Materials and methods

Animals

$Crx^{-/-}$ mice were provided by Dr. Connie Cepko. The $Crx^{-/-}$ mice were made on a 129sv background (Furukawa *et al.* 1999); 129sv mice were used as wild-type controls in the current study. Genotypes were identified by use of primers detecting *Crx* (Table 1) and primers detecting the *neo* cassette in $Crx^{-/-}$ mice, which amplify a 470-bp fragment not detected in the wild-type mouse. Wild-type and $Crx^{-/-}$ mice (male and female, > 2 months of age) were bred and kept in a 12L : 12D light cycle with food and water *ad libitum*. To collect tissue, animals were killed with CO_2 and decapitated at zeitgeber time (ZT) 6 or ZT20. The skull cap was carefully removed with the intention not to alter the position of the superficial pineal gland. The gland was then located on the skull, removed and cleaned of extraneous tissue. Dim red light was used when animals were euthanized at ZT20. For microarray analysis, pineal glands were immediately frozen on dry ice, then stored at −80°C in pools of eight glands; for qRT-PCR, pools of 3–8 glands were prepared similarly. For radiochemical *in situ* hybridization histology, brains from five wild-type animals and five $Crx^{-/-}$ animals were used. For Affymetrix GeneChip analysis and qRT-PCR, three pools were analyzed per time point. All animal experiments were performed in accordance with the guidelines of EU Directive 86/609/EEC (approved by the Danish Council for

Table 1 Primer sequences for genotyping, quantitative real-time RT-PCR analyses and cloning. Where accession numbers are not available, Entrez Gene identifiers are given

Gene	GenBank accession no.	Position	Forward primer 5′-3′	Reverse primer 5′-3′
Genotyping				
Crx	NM_007770.4	461–770	GCAGCGACAGCAGCAGAAACA	ATGACCTATGCCCCGGCTTCT
Neo[a]	–	274–975	ATGGATTGCACGCAGGTTCTC	GATCTGGACGAAGAGCATCAG
qRT-PCR				
Aanat	NM_009591.3	4–105	TGCAGTCAGGAGTCTCAGCTT	AAGTGCTCCCTGAGCAACAG
Actb	NM_007393.2	414–550	CTAAGGCCAACCGTGAAAAG	GTCTCCGGAGTCCATCACAAT
Ap1g1	NM_009677.3	2489–2641	GAGCTAGACATGACGGACTTTG	CAGCTGTTGCTTCTGTGGAT
B2m	NM_009735.3	111–211	TATCCAGAAAACCCCTCAAAT	GAGGCGGGTGGAACTGTGTTA
Cpm	NM_027468	984–1139	AAGTGTTCGATCAGAGTGGAGC	CGTGTCCAGGGACTGTAACAT
Cry1	NM_007771	872–1015	TCAATTGAGTATGATTCTGAGCCT	TCCGCCATTGAGTTCTATGATC
Gapdh	XM_001473623.1	77–178	TGGTGAAGGTCGGTGTGAACG	AGGGGTCGTTGATGGCAACAA
Gm626	268729	319–473	GGGAGCGAGAGTGACTGG	GATGAAAATCAGCTGAGGGC
Gng4	NM_010317	210–368	GGAGTGCAGGAATGAAGGAA	GCACGTGGGCTTCACAGTA
Hoxc4	NM_013553	1726–1867	GGAGGACAGCAAACAAAGCTA	TAACCACGATGAGGGTAGGG
Pde10a	NM_011866	372–518	GAAGGCTGACCGAGTGTTTC	TGGTTTTCCTCTTCAGCCAC
Pvr	NM_027514	454–606	TTCCCCAGAGGCAGTAGAAG	AGAGATTCGTCCAGGAGGGT
Rasgrf 1	NM_011245	1757–1911	GAGGGCTGTGAGATCCTCCT	GAATAGGAAACACTGGCGCT
Rps27a	NM_024277	232–376	GAAGACCCTTACGGGGAAAA	GCCATCTTCCAGCTGCTTAC
Snap25	NM_011428.3	93–244	GCTCCTCCACTCTTGCTACC	GCTCATTGCGCATGTCTGCG
Ube2d2	NM_019912.2	389–533	GGCTCTGAAGAGAATCCACAA	TACTCCACCCTGATAGGGGC
1700042O10RIK	73321	836–988	CTGCATAGATTTTGCACGGA	TAACTGAGGGTTGATTGGGG
PCR for cloning				
Hoxc4	NM_013553	61–465	GGTGTGCAATGGTGAGCACC	TGGAATCCCGATTCCCTGGT
Hoxc4	NM_013553	351–965	TGGACTCTAACTACATCGAT	TCTTCCATTTCATACGACGGT
Hoxc4	NM_013553	861–1867	ACCGCTACCTGACCCGAAGG	TAACCACGATGAGGGTAGGG

[a]The primers generate a Neo derived product within this area.

Animal Experiments) and the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Microarray

RNA was purified using the RNeasy micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol (including DNase treatment); high quality was verified using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). A commercially available kit (Nugen Ovation RNA amplification system V2, NugenTech, San Carlos, CA, USA) was used to convert 100 ng RNA to cDNA following the manufacturer's protocol. Double-stranded cDNA was SPIA® amplified using the same kit followed by purification (instead of using the washing buffer, the cDNA was washed twice in freshly prepared 80% ethanol) (Zymo Research, Orange, CA, USA). cDNA (3.75 μg) was used for fragmentation and labeling (FL-Ovation cDNA Biotin Module V2, NugenTech). Labeled cDNA was mixed with control oligonucleotides B2, 20× eukaryotic hybridization controls (Affymetrix, Santa Clara, CA, USA), herring sperm DNA, acetylated BSA, 2× hybridization buffer, and DMSO according to the Affymetrix protocol. This was used for analysis with the Affymetrix mouse GeneChip 430_2 microarray chip. The labeled cDNA was allowed to hybridize for 18–24 h at 45°C before processing according to Affymetrix protocols. GeneChips were scanned on an Affymetrix 3000 Scanner. The microarray data are available at the Entrez Gene Expression Omnibus, National Center for Biotechnology (Edgar *et al.* 2002), and are accessible through GEO series accession no. GSE24625 (ncbi.nlm.nih.gov).

Data analysis

The data files (.CEL) were analyzed with ChipInspector V2.0 software (Genomatix Software Inc., Munich, Germany); using this program gene expression in wild-type glands at ZT6 (n = 3 groups) was compared with that in wild-type glands at ZT20 (n = 3 groups). $Crx^{-/-}$ ZT6 (n = 3 groups) and $Crx^{-/-}$ ZT20 (n = 3 groups) were compared in a similar manner. In addition, a comparison of wild-type (n = 6 groups) and $Crx^{-/-}$ (n = 6 groups) gene expression was done independently of sampling time. In all analyses, the following filters were chosen: exhaustive matching, a false discovery rate = 0, cut off = 1, region size = 300 bp and a minimum of 4 and 5 significant probes (depending on the number of genes). Differences of $p < 0.05$ were considered to be statistically significant.

Bioinformatics

Networks and canonical pathways were identified using Ingenuity Pathway Analysis (IPA; Ingenuity Systems, Redwood City, CA, USA). Analysis of consensus sequences and transcriptional function were performed with Genomatix Pathway System (GePS), Genomatix Gene2Promoter and Genomatix RegionMiner.

Quantitative real-time RT-PCR analysis

RNA was purified using an RNeasy micro kit (Qiagen, Copenhagen, Denmark) according to the manufacturer's protocol. cDNA was synthesized from 350 ng of DNase-treated RNA using random primers and SuperScript III (Invitrogen, Taastrup, Denmark). Quantification of each gene was performed by use of a gene-specific internal standard curve of known copy number. For quantification, standard curves were produced by serial dilution (10^1–10^7 copies/μL) of each PCR target; target sequences had been cloned into a plasmid (for details see molecular cloning). Standard curves were prepared for each qRT-PCR analysis.

qRT-PCR (LightCycler 1.5; Roche, Hvidovre, Denmark) reactions were carried out in a 20 μL volume with 1 μM primers (for sequences see Table 1), 1× SYBR green master mix (SABiosciences, Copenhagen, Denmark) and a 2-μL sample of a 3.5-fold dilution of cDNA. The program included an initial step of 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 30 s, extension at 72°C for 30 s. Product specificity was confirmed initially on an agarose gel, thereafter by melting curve analysis after every qRT-PCR run.

Housekeeping gene selection for normalization was performed using 2 μL of 3.5-fold diluted samples of cDNA from wild-type and $Crx^{-/-}$ pineal glands. Seven genes (*Gapdh*, *Ap1g1*, *β-actin*, *B2m*, *Snap25*, *Rps27a* and *Ube2d2*) were analyzed to select those which exhibited the smallest time-dependent difference in expression. Crossing points were used for analysis by GeNorm (Vandesompele *et al.* 2002). The genes selected were *Gapdh*, *Snap25*, *Ap1g1 and Ube2d2*.

Data analysis

Two-way ANOVA was used to test for the influence of Crx on the genotype using GraphPad Prism V4 (GraphPad software). The four conditions were (129sv ZT6, 129sv ZT20, $Crx^{-/-}$ ZT6 and $Crx^{-/-}$ ZT20) compared with respect for genotype.

For each genotype, Student's two-tailed t-test with Welch's correction was used on log(2) transformed data to determine if genes were differentially expressed at a day/night basis. For each time point, the data are presented as the mean and standard error of mean (SEM) of three samples. A p-value of < 0.05 was considered to represent a statistically significant difference.

Molecular cloning of standards used for qRT-PCR and of overlapping fragments of *Hoxc4*

Target sequences for molecular cloning were generated by standard PCR reactions using pineal cDNA prepared from DNase-treated RNA (primer sequences are listed in Table 1). PCR products were isolated by gel electrophoresis and gel extraction (Qiagen, Sollentuna, Sweden) and cloned into pGEM-T Easy vectors (Promega, Madison, WI, USA) according to the manufacturer's instructions. Plasmids were selected and amplified in DH5-α cells (Invitrogen, Hvidovre, Denmark). In all cases, insert identity was confirmed first by EcoRI digestion and agarose gel analysis followed by sequencing (DNA Technology, Århus, Denmark). The qRT-PCR products ranged in size from 101 to 159 bp.

Transmission electron microscopy

The pineal glands from both $Crx^{-/-}$ mice and wild-type mice were fixed by immersion in cold 4% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 60 min. The sections were then dehydrated in a series of ethanol (30%, 50%, 70%, and 96%), block-stained in 1% uranyl acetate in absolute ethanol for 1 h, rinsed twice in absolute ethanol, and embedded by propylene oxide in Epon®. Two-micrometer-thick survey sections were cut and counter-stained with toluidine blue. Ultrathin sections, with a gray interference color, were cut from pre-selected areas and post-

stained in uranyl acetate and lead citrate. They were then viewed in a Philips EM 208 transmission electron microscope operated at 80 kV.

Radiochemical *in situ* hybridization histological detection of *Hoxc4* transcripts

Cryostat sections (12 μm) were mounted on Superfrost Plus slides and hybridized as previously described (Møller *et al.* 1997; Rath *et al.* 2007) with a mixture of three ^{35}S-labelled 38-mer antisense DNA probes directed against mouse *Hoxc4* mRNA (NM_013553.2): 5′-CATAAAGCCCTCCTACTAGCTAGCGACC CTGTAAAGTT-3′ (278–241), 5′-CGAATTGCCAGGCCCCTGG AGACTGGTGCAGCTATACT-3′ (562–525), 5′-TTCACCCAAA CCAGACCATCACACCTTGCAATATATAA-3′ (1519–1482). Sections from five wild-type and five $Crx^{-/-}$ mice were prepared. The hybridized sections were exposed to X-ray film for 3 weeks and developed.

Results

Morphology of the pineal gland

Examination of the $Crx^{-/-}$ pineal (Fig. 1) revealed that the morphology is not different from the wild-type gland. Rather, the gland exhibits normal features, evident from light and electron microscopic examination, characterized by a perivascular space with myelinated nerve fibers and interstitial cells surrounded by a parenchyma of pinealocytes; the shape, density and organelle content of pinealocytes in the $Crx^{-/-}$ and wild-type pineal gland (Upson *et al.* 1976; Møller *et al.* 1978) are indistinguishable.

Crx has broad modulatory effects on gene expression in the pineal gland

Comparison of gene expression in the wild-type and $Crx^{-/-}$ pineal gland indicated that 512 genes were significantly ($p < 0.05$) down-regulated in $Crx^{-/-}$ pineal gland: 23 > 8-fold, 65 4-to 8-fold (Table 2) and 424 2- to 4-fold (Table S1). In addition, 714 genes were significantly ($p < 0.05$) up-regulated in the $Crx^{-/-}$ pineal gland: 10 > 8-fold, 33 4-to 8-fold (Table 2), and 671 2-to 4- fold (Table S2).

Among the genes down-regulated in the $Crx^{-/-}$ mouse pineal gland, the most affected gene other than *Crx* was *Cbln1*, which decreased by nearly 20-fold; *Cbln1* encodes a protein that releases norepinephrine via an adenylate cyclase/PKA-dependent signaling pathway (Albertin *et al.* 2000).

The most highly up-regulated transcript in the $Crx^{-/-}$ pineal gland was identified as AK140080, which maps to a location adjacent to the ras-GTPase-activating protein SH3 domain binding protein (*G3bp1*) and is therefore assumed to be the 3′-extension of this gene. The second most up-regulated gene was *Hoxc4* (∼18-fold). *Hoxc4* encodes a homeodomain transcription factor required for development of the oesophagus and spinal cord (Geada *et al.* 1992).

Analysis of networks by IPA (Ingenuity Systems) revealed that Crx influences genes involved in cellular assembly and organization, signaling and cell morphology (Table 3), among others. The indication that Crx affects genes involved in morphology is interesting because, as indicated above, the morphology and cell composition of the $Crx^{-/-}$ pineal gland appears normal (Fig. 1). Along with the ephrin receptor pathway, the GABA pathway was also observed to be affected by deletion of *Crx*.

An *in silico* analysis with Genomatix software revealed that among the 1288 genes that were dysregulated more than 2-fold in the $Crx^{-/-}$ mouse, 433 (represented by 772 transcripts) have predicted binding affinity for Crx (core consensus, TAATC) in the 500 bp promoter region upstream of the first transcriptional start site. Analysis of the genes regulated > 4-fold with a Crx consensus sequence, revealed that nine had a transcriptional regulatory function (Table 2). This suggests that some effects seen in the $Crx^{-/-}$ pineal gland might be mediated by changes in one or more of these transcription factors.

Crx and Otx2 consensus sequences are similar and belong to the photoreceptor conserved element (PCE) family (Kiku-

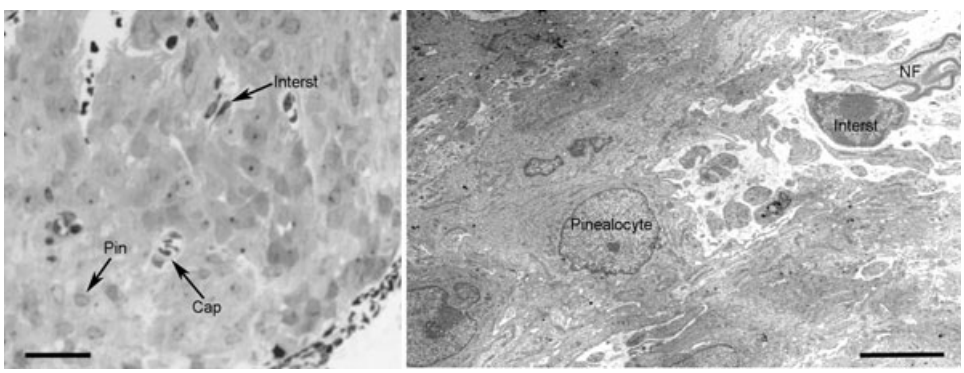

Fig. 1 Transmission electron microscopic analysis of the pineal gland of the $Crx^{-/-}$ mouse. (left) A Epon-embedded section (2 μm) of a $Crx^{-/-}$ mouse pineal tissue stained with Toluidine blue. The gland exhibits a normal morphology with pinealocytes (Pin), interstitial cells (Interst) and capillaries (Cap). Bar = 25 μm. (right) Electron micrograph of the pineal gland of the $Crx^{-/-}$ mouse. A perivascular space with a myelinated nerve fiber (NF) and interstitial cell (Interst) is seen surrounded by pinealocytes with normal shape, density and organelle content. Bar = 5 μm.

Table 2 Genes dysregulated in the pineal gland of the *Crx*$^{-/-}$ mouse. The pineal transcriptomes of the wild-type and *Crx*$^{-/-}$ were compared independent of time of sampling. A set of 512 genes were found to be directly or indirectly down-regulated by Crx deletion ($p < 0.05$); among these, 424 genes are down-regulated only 2- to 4-fold; these genes are listed in Table S1. A set of 714 genes were found to be directly or indirectly up-regulated by Crx deletion ($p < 0.05$); among these, 671 genes are up-regulated only 2- to 4-fold; these genes are listed in Table S2. All genes are assigned by ChipInspector V2 (Genomatix). A '+' symbol in the Crx, Otx2 and/or Hoxc4 columns indicate the gene has at least one CRX, OTX2, or HOXC4 consensus sequence in the promoter region (500 bp upstream of first TSS). Genes encoding transcription factors are indentified by a '+' symbol in the column labeled TF. The 1/*x* convention is used to indicate down-regulation by a factor of *x*. For further details, see the Materials and methods section

Gene Symbol	Fold	Crx	Otx2	Hoxc4	TF
G3bp1	26.4				
Hoxc4	17.5				+
Smtnl2	15.6	+			
Tia1	15.5	+	+	+	
D430019H16Rik	13.5				
Chodl	12.0			+	
A330092A19Rik	10.3				N/A
Glra1	9.0				
Mpp3	9.0	+	+		
Kcnip4	8.2	+		+	
Nefl	6.5				
C230022P04Rik	6.5				N/A
Ipcef1	5.9				
Trank1	5.8	+		+	
Isl1	5.7	+			+
Kctd4	5.6	+	+		
Lgi2	5.5		+		
Neurod2	5.5	+			+
Ngef	5.5				
Gria2	5.4	+			
Caln1	5.1	+		+	
Tmem178	5.0	+		+	
Synpr	5.0	+		+	
A2bp1	4.9			+	
Grin2c	4.9				
Prox1	4.9	+		+	
St18	4.8				+
AI593442	4.8				
Crtam	4.7	+			
Cntn3	4.5	+	+	+	
Epha8	4.5				
Dusp26	4.4				
Hist1h3i	4.4				
Pou3f1	4.4				+
LOC100045707	4.4				
Atp13a5	4.4	+			
Kcnq2	4.4	+			
Cox6a2	4.3				
Slc6a1	4.3				
2900092D14Rik	4.3				
Lrtm1	4.2	+	+		
Nova1	4.1	+		+	
Nefm	4.1				
Cbln1	1/19.4				
1700042O10Rik	1/19.3	+			
4833423E24Rik	1/17.4	+			
Gm626	1/16.0	+	+	+	
Gm2595	1/16.0				N/A
Ptpn20	1/15.7	+			
Tal2	1/12.2				+
Mycn	1/12.1				+
Tubb3	1/12.0				
Hk2	1/11.5	+			
Ankrd33	1/11.2				
Trim15	1/10.5				
Camkv	1/10.3	+			
Ng23[a]	1/9.9				
Rasgrf1	1/9.2	+			
Myog	1/8.9	+			+
Cyp2j13	1/8.8	+			
Odz2	1/8.8				
Panx2	1/8.6				
Rho	1/8.6	+			
17H6S56E-3	1/8.5				
Accn3	1/8.2	+	+	+	
Rpp25	1/8.1				
Gabrd	1/7.9				
Mst1r	1/7.9	+			
Arhgef15	1/7.7	+	+		
4930583H14Rik	1/7.6	+			
Gpnmb	1/7.4				
Syngr3	1/7.4				
Papss2	1/7.1	+		+	
3930402G23Rik	1/7.1				
Piwil4	1/6.8	+		+	
Slc24a1	1/6.7				
Rpia	1/6.5				
Ube2t	1/6.3	+		+	
LOC100047829	1/6.3				
Fhod3	1/6.1	+		+	
Nefh	1/6.1				
Gm2694	1/6.1				N/A
LOC100045304	1/6.1				
FLJ22297	1/6.1				N/A
FLJ22717	1/6.1				N/A
Rbpms	1/5.9	+			
Garnl3	1/5.8	+	+	+	
Mypn	1/5.8	+		+	
Npl	1/5.7	+	+		
Stc2	1/5.7				
Wdr66	1/5.6				

Table 2 (*continued*)

Gene Symbol	Fold	Crx	Otx2	Hoxc4	TF
Recql	1/5.5	+			
Ret	1/5.4				
Adcy3	1/5.3				
Sall4	1/5.3				
2310011E23Rik	1/5.3				
Als2	1/5.2	+	+	+	
Mpp4	1/5.2				
Mesp1	1/5.1			+	+
Cpm	1/5.0	+	+	+	
Golt1b	1/5.0				
Pvr	1/5.0	+		+	
C1ql3	1/4.9				
Gfra1	1/4.9				
2010107G12Rik	1/4.7	+	+		
Cenpo	1/4.6	+	+		
Gabrr1	1/4.6	+			
Lhfpl5	1/4.6				
Rnf207	1/4.6				
LOC639211	1/4.6				
Accn1	1/4.5	+			
Bub1b	1/4.5		+	+	
Galntl2	1/4.5	+			
Sez6l	1/4.5	+			
Gng4	1/4.4				
Sel1l3	1/4.4				
Spsb4	1/4.3	+	+		
A930007K23Rik	1/4.3				N/A
D4Bwg0951e	1/4.3	+	+		
Acoxl	1/4.2	+	+	+	
Gngt1	1/4.2		+	+	
Lrit1	1/4.2	+			
Akap6	1/4.1			+	
Bace2	1/4.1	+	+		
Bdkrb2	1/4.1				
Dntt	1/4.1				
Kif22	1/4.1	+			
Mc1r	1/4.1				
Pde6g	1/4.1	+			
Rgs20	1/4.1		+	+	
LOC100044395	1/4.1				

N/A, information is not available.

[a]Gene name assigned by authors based on homology.

Table 3 Networks of genes and canonical pathways influenced by the deletion of Crx. Network and canonical pathway analysis was done using Ingenuity IPA software on the 50 most up- and 50 most down-regulated genes. *p*-value < 0.05

Networks	Canonical pathways
Cell-to-cell signaling and interaction	Amyotrophic lateral sclerosis signaling
Cellular assembly and organization	GABA receptor signaling
Cellular function and maintenance	Ephrin receptor signaling
Neurological disease	Neuropathic pain signaling in dorsal horn neurons
Genetic disorder	
Nutritional disease	
Lipid metabolism	
Molecular transport	
Small molecule biochemistry	
Nervous system development and function	
Cellular compromise	
Organismal injury and abnormalities	

chi *et al.* 1993). It was found that 194 of the dysregulated genes (representing 294 transcripts) had an Otx2 regulatory sequence (core consensus TAATCC/T) but only 54 genes could theoretically be regulated by both Crx and Otx2, thereby providing *in silico* evidence that expression of these genes might reflect the direct action of these transcription factors (Table 2). *Aanat* was one of these genes, having consensus sequences for both Crx and Otx2, as previously reported (Li *et al.* 1998). It was also found that in some cases one gene encodes two transcripts, only one of which has a Crx and/or Otx2 regulatory sequence, whereas the other does not; this is seen with *Tia1* and *Mpp3*, and may reflect use of alternative start sites.

The finding of a large up-regulation of the transcription factor *Hoxc4* encouraged us to investigate *in silico* if some of the most dysregulated genes in the $Crx^{-/-}$ mouse had regulatory sequences for Hoxc4 (core consensus TAATTA). Three hundred and fifteen transcripts, encoded by 205 different genes, were found to have an Hoxc4 consensus sequence. None of the transcripts that were up-regulated > 4-fold had a transcriptional role, thereby providing no reason to suspect that effects of Hoxc4 were mediated by any transcription factor. The Hoxc4 co-cited network generated by Genomatix Pathway System (GePS)(Fig. 2) has only a single gene that is dysregulated in the $Crx^{-/-}$ pineal gland, Hoxc5 (2-fold). The absence of any other dysregulated gene in this co-cited network suggests that the genes influenced by Hoxc4 in the pineal gland differ from those regulated by Hoxc4 in other tissues.

Crx influences differential day/night expression of genes in the mouse pineal gland

In the wild-type pineal gland, 51 transcripts were differentially expressed on a night/day basis (> 2-fold, $p < 0.05$, Table 4); amongst these the abundance of 34 (67%) decreased at night and that of 17 (33%) increased at night (Table 4). The abundance of ten transcripts changed more than 8-fold and that of 35 transcripts changed 4- to 8-fold

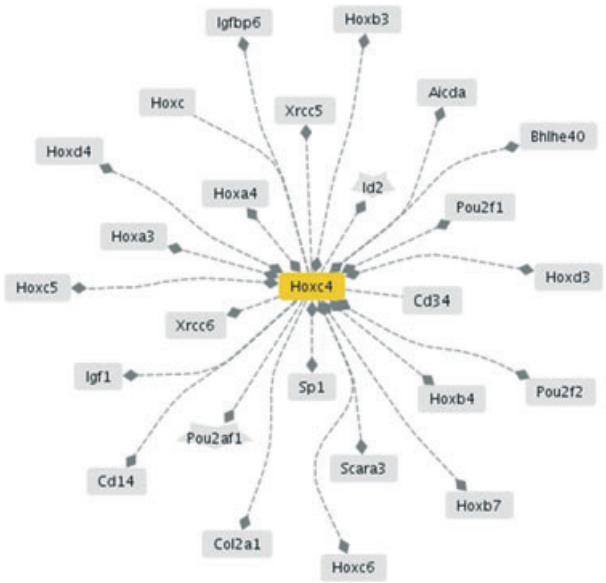

Fig. 2 Genes co-cited with Hoxc4. The dashed line indicates co-citation of the connected genes in the literature; a diamond indicates the promoter of gene B (the gene with the diamond) has a consensus sequence for the transcription factor encoded by gene A. An additional filled diamond at the other end of the line means that the promoter of gene A has a consensus sequence for the transcription factor encoded by gene B. The only gene from this network that exhibited a change in expression in the *Crx*$^{-/-}$ mouse other than *Hoxc4* is *Hoxc5* (2-fold). (figure generated by the Genomatix Pathway System).

(Table 4); the transcript with the largest nocturnal increase was *Aanat*. The transcript exhibiting the largest nocturnal decrease (1/9.2) was encoded by *Nrxn3*, which is thought to be involved in synaptic plasticity (Kelai *et al.* 2008).

In the *Crx*$^{-/-}$ mouse, 49 transcripts displayed differential night/day expression (Table 4), of which seven are not annotated. Among these 49 differentially expressed transcripts, 22 (45%) decreased at night and 27 (55%) increased at night (Table 4). The transcripts that were day/night expressed in the *Crx*$^{-/-}$ pineal gland differed from those in the wild-type mouse; only eight genes had a different day/night expression in both groups (Table 4).

Of special note was the observation that *Aanat* increased in the *Crx*$^{-/-}$ mouse pineal at night compared with day time (~8.5-fold; Table 4). The transcript that exhibited the largest decrease at night was *Nr1d1*/*Rev-erbα*, which functions as a transcriptional repressor and plays a role in circadian systems (Burris 2008; Meng *et al.* 2008).

Genes that were differentially expressed on a day/night basis in the wild-type mouse were associated with networks dedicated to behavior, development and neural function as defined by IPA; the primary canonical pathways include dopamine receptor signaling, GABA receptor signaling and β-adrenergic signaling (Table 5), the last two of which are

Table 4 Transcripts differentially expressed ($p < 0.05$) on a day/night basis in the wild-type and/or *Crx*$^{-/-}$ pineal gland. Ratios are given as transcript levels at ZT20/ZT6. The columns labeled Crx, Otx2 and Hoxc4 indicate the presence of regulatory binding sequences for the indicated transcription factor in the promoter of the gene (500 bp upstream of first TSS)

Gene symbol	Wild-type	*Crx*$^{-/-}$	Crx	Otx2	Hoxc4
Genes up-regulated at night					
Aanat	14.6	8.5	+	+	
Gng4	6.9	6.9			
Slc6a5	7.4	5.0	+		
E2f8	13.5	–	+		
Evi2a	12.0	–	+		
Cpm	11.2	–	+	+	+
Pols	9.5	–			
Gm2788	9.3	–			
LOC100046261	9.3	–			
2810011L19Rik	9.2	–			
Pvr	8.9	–	+		+
3930402G23Rik	7.5	–			
Kcnc1	7.5	–	+		
Asphd2	7.4	–			
E130002L11Rik	6.0	–	+		
Gulo	5.7	–			
Frmpd1	5.6	–	+		
Pde10a	–	4.7	+		+
Lrit1	–	4.4	+		
Mitf	–	4.3	+	+	
Syt10[a]	–	4.2			
Vil1	–	4.1			
Dclk1	–	4.0	+		+
ENSMMUT00000011414	–	3.7			
Ccdc109b	–	3.7		+	+
EG245190	–	3.7			
Rftn1	–	3.7	+		
Slc6a17	–	3.6			
Gls2	–	3.5			
Rho	–	3.4	+		
Odc1	–	3.4			
Adora1	–	3.2	+		
1810041L15Rik	–	3.2		+	
Clec4d	–	3.1	+		+
Bok	–	2.9	+		+
Nrp2[a]	–	2.8			
Cry1	–	2.8	+		+
6430411K18Rik	–	2.6		+	
Clstn3	–	2.5			
Stard4	–	2.3			
Genes down-regulated at night					
Cacnb2	1/7.4	1/2.9	+		+
Pdc	1/5.9	1/3.7	+		
Hspa12a	1/4.8	1/3.3			
Nr1d1	1/4.4	1/5.9		+	
Atg16l1	1/4.0	1/2.6	+	+	
Nrxn3	1/9.2	–	+		
AK048867	1/6.6	–	+		

Table 4 (*Continued*)

Gene symbol	Wild-type	*Crx*$^{-/-}$	Crx	Otx2	Hoxc4
Cntn4	1/6.0	–	+	+	+
Xpo7	1/5.9	–			
Gabrd	1/5.7	–			
BC027072	1/5.5	–	+		
Clca3	1/5.4	–	+		
A330008L17Rik	1/5.4	–			
A730054J21Rik	1/5.4	–			+
Ppp2r2b	1/5.3	–	+	+	+
ENSPTRT00000066164	1/5.3	–			
Car8	1/4.9	–			
Ppfia2	1/4.9	–	+	+	+
LOC676792	1/4.9	–			
Ubr1[a]	1/4.8	–			
EG665934	1/4.8	–			
Bai3	1/4.6	–	+		+
Gabra1	1/4.5	–	+		+
Fat3	1/4.4	–		+	
Slc8a1	1/4.4	–	+	+	+
Csmd3	1/4.3	–	+	+	+
4930414L22Rik	1/4.3	–			
Fmn1	1/4.2	–	+		+
Mina	1/3.9	–	+	+	+
Sphkap	1/3.9	–			
Sv2b	1/3.9	–		+	+
4930526L06Rik	1/3.9	–			
Ddc	1/3.8	–	+		+
Tmem90a	1/3.7	–			
Cdh8	–	1/4.0	+		
Gngt1	–	1/4.0		+	+
Rprm	–	1/3.8			
Chrna6	–	1/3.7	+		
Adcy1	–	1/3.3			
Sgip1	–	1/3.1	+		+
ENST00000371039	–	1/3.1			
Sphk2	–	1/3.0			
Dbp	–	1/2.9	+		
A830039N20Rik	–	1/2.6	+		
C030014L02	–	1/2.6			
B3galt2	–	1/2.5			+
Cngb3	–	1/2.4	+	+	
Gjd2	–	1/2.4	+		
Mypn	–	1/2.4	+		+
Srpk3	–	1/2.3			+
Morn1	–	1/2.0			

[a]Gene name assigned by authors based on homology.

[–]Transcript is not differentially expressed on a day/night basis. For further details, see the Materials and methods section.

involved in melatonin synthesis (Klein *et al.* 1971; Sun *et al.* 2002).

The genes that are differently expressed on a day/night basis in the *Crx*$^{-/-}$ mouse were found to be involved in networks committed to behavior, development and neural function; the same networks as observed in the wild-type mouse (Table 5). Different canonical pathways were observed to be altered in the *Crx*$^{-/-}$ mouse versus the wild-type animal: the phototransduction pathway, protein kinase A signaling, cAMP-mediated signaling and circadian rhythm signaling exhibit daily changes in the *Crx*$^{-/-}$ mouse (Table 5). These pathways are all involved in the production of melatonin or the control of this process. The β-adrenergic signaling pathway was the only canonical pathway to exhibit differential night/day expression in the pineal glands of both the wild-type and *Crx*$^{-/-}$ mice (Table 5).

Using Genomatix software, it was found that 24 of the 49 genes exhibiting differential day/night expression in the *Crx*$^{-/-}$ mouse had a Crx consensus sequence (Table 4), only five genes had binding affinity for both Crx and Otx2. Six of the up-regulated genes had a Hoxc4 consensus sequence, indicating that these are candidates for up-regulation by Hoxc4. Moreover, it is possible that the transcription factor Cry1, might mediate effects of Hoxc4 by virtue of a Hoxc4 consensus sequence in the *Cry1* promoter. In addition, it was found that the *Aanat* 500 bp upstream promoter did not have a Cry1 consensus sequence indicating that Cry1 is not responsible for the up-regulation of *Aanat* at night in the *Crx*$^{-/-}$ mouse in the absence of Crx.

qRT-PCR validation of microarray data

Six transcripts that exhibited differential night/day expression by microarray in either wild-type or *Crx*$^{-/-}$ pineal glands (*Aanat*, *Gng4*, *Cpm*, *Pvr*, *Pde10a*, *Cry1*) were examined with qRT-PCR; a daily rhythm was detected in all encoded transcripts in glands from wild-type animals (Fig. 3) and in five genes from *Crx*$^{-/-}$ animals. In addition, the differential wild-type /*Crx*$^{-/-}$ expression patterns seen in four transcripts (*Hoxc4*, *1700042O10Rik*, *Gm626* and *Rasgrf1*) were confirmed by qRT-PCR.

In the case of *Pde10a*, a set of primers directed against the 3′ region of the transcript confirmed the results of microarray which interrogated this region (Fig. 3). However, a set of primers directed against the 5′ region did not confirm the results of microarray; rather qRT-PCR with these primers revealed a 1/3.5-fold ZT6/ZT20 inverse rhythm (6.2×10^3/1.8×10^3 transcripts) in the wild-type (p-value = 0.02) and 1/1.4-fold ZT6/ZT20 inverse rhythm (3.8×10^3/2.7×10^3) in the *Crx*$^{-/-}$ mouse (p-value = 0.1), indicating at least two *Pde10a* transcripts are expressed in the pineal gland and that they are differentially regulated.

Studies on *Hoxc4*

The marked effects of *Crx* deletion on *Hoxc4* was studied in greater detail because homeobox genes are known to broadly control cascades of gene expression. Three overlapping portions of the Hoxc4 transcript were cloned from *Crx*$^{-/-}$ pineal mRNA; sequencing confirmed their identity (open

Table 5 Assignment of genes differentially expressed on a day/night basis in the wild-type and *Crx*$^{-/-}$ mouse to networks and canonical pathways. Network and canonical pathway analysis was performed using Ingenuity IPA software. *p*-value < 0.05

	Networks	Canonical pathways
Wild-type	Behavior	Cardiac b-adrenergic signaling
	Nervous system development and function	GABA receptor signaling
	Gastrointestinal disease	Dopamine receptor signaling
	Neurological disease	
	Psychological disorders	
	Cell morphology	
	Cellular development	
	Tumor morphology	
	Lipid metabolism	
	Small molecule biochemistry	
	Cell death	
	Organ morphology	
Crx$^{-/-}$	Behavior	Phototransduction pathway
	Nervous system development and function	Protein kinase A signaling
	Genetic disorder	cAMP-mediated signaling
	Cell-to-cell signaling and interaction	Circadian rhythm signaling
	Cardiovascular disease	Cardiac b-adrenergic signaling
	Neurological disease	
	Skeletal and muscular disorders	
	Cellular development	
	Cellular growth and proliferation	
	Embryonic development	
	Organismal development	
	Skeletal and muscular system development and function	

reading frame > 99% identical to NM_013553.2), thereby indicating that a full-length *Hoxc4* transcript is likely to be present in the *Crx*$^{-/-}$ pineal gland.

Hoxc4 expression in the *Crx*$^{-/-}$ mouse pineal gland was also confirmed using radiochemical *in situ* hybridization histology (Fig. 4), which failed to detect expression of the gene in surrounding tissues or in the wild-type gland.

Discussion

This study was primarily designed to extend previous studies on the pineal gland of the *Crx*$^{-/-}$ mouse; these results, including the broad modulatory effects of Crx on the pineal transcriptome, will be addressed below. In addition, because this is the first report of microarray analysis of the adult mouse pineal gland, the results of this study provide new understanding of gene expression in this tissue; this topic will also be discussed. It should be noted that microarray has been used to study the neonatal mouse pineal gland (Munoz *et al.* 2007). There are large differences in the genes expressed in the neonatal and adult pineal gland, consistent with evidence that marked developmental changes occur at about this time in the rodent, including a marked decrease in cell division and an increase in expression of genes linked to melatonin synthesis and visual signal transduction (Quay and Levine 1957; Quay 1974; Klein *et al.* 1981). However, a thorough discussion of the developmental differences in gene expression in the pineal gland as revealed by both microarray studies is beyond the scope of this report.

Night/day differences in transcript abundance in the pineal gland are less prevalent in the mouse as compared with the rat

The pineal transcriptome has previously been studied by microarray in the rat (Tosini *et al.* 2008; Bailey *et al.* 2009), chicken (Bailey *et al.* 2003) and zebrafish (Toyama *et al.* 2009) but not in the mouse. Here we have found that 51 genes in the wild-type mouse pineal gland exhibit a daily rhythm (> 2-fold). These 51 genes were found to be elements of canonical pathways that impact melatonin synthesis (Table 5). In a similar study of the rat pineal, Bailey *et al.* (2009) found > 600 genes were different day/night expressed > 2-fold. The rat versus mouse difference in the number genes differentially expressed on a night/day basis is striking. Furthermore, the amplitude of changes appears to be greater in the rat, in which 130 genes exhibited a > 4-fold night/day difference. In the wild-type mouse, there are only 35 genes with a night/day difference of this magnitude; in addition, only *Aanat* and *Pvr* show a difference in both rat and mouse.

Differences in day/night gene expression between the rat and mouse may reflect fundamental biological differences between these species as are known to exist between other

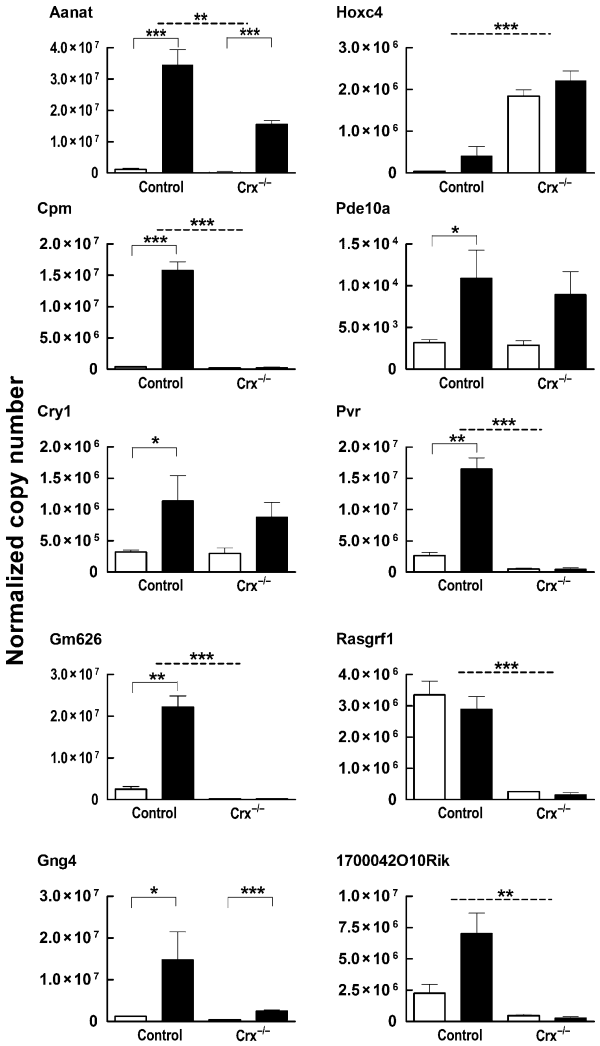

Fig. 3 qRT-PCR analysis of transcripts detected as either day/night expressed or differentially expressed in $Crx^{-/-}$ mice as compared with wild-types. Black bars, night samples; white bars, day samples. Transcripts are identified by gene symbols. Values were normalized to *Gapdh*, Ube2d2, *Snap25* and *Ap1g1*. Each value represents the mean ± SEM of three independent analyses. **p*-values < 0.05; ***p*-values < 0.01; and, ****p*-values < 0.001. For further details, see the Materials and methods section.

mammals. For example, the large night/day difference in *Aanat* transcript number seen in the rat is not seen in the Rhesus monkey or sheep, which exhibit little or no night/day difference in AANAT mRNA whereas changes in AANAT activity do occur (Coon *et al.* 1999, 2002; Johnston *et al.* 2004; Klein 2007), presumably as a function of post-translational mechanisms (Klein 2007).

Inbreeding might also be responsible for the small number of genes expressed differentially on day/night basis in the mouse pineal gland. This is consistent with the observation that the genes encoding the melatonin synthesis enzymes Aanat and Asmt/Hiomt are non-functional in most laboratory mice because of mutations. Accordingly, a global difference in the daily pattern of gene expression might be caused by a mutation in a gene with a broad impact on gene expression, for example, a transcription-related factor.

These findings provide further reason to question whether conclusions drawn from analysis of gene expression patterns in tissues of one species are necessarily applicable to other species.

The broad pleiotropic effect of Crx on the pineal transcriptome

The current study identified more than a thousand genes that are dysregulated in the $Crx^{-/-}$ mouse, more than half of which were up-regulated. It is surprising that differential expression of such a high number of genes is not reflected in a major morphological change in the pineal gland (Fig. 1) as for example seen in retina of this mouse. It is of interest that several of the dysregulated transcripts in the $Crx^{-/-}$ mouse are part of the ephrin pathway, which controls cell morphology and cell communication; in view of this, it is surprising that morphological changes are not apparent.

An *in silico* analysis of Hoxc4 consensus sequences revealed that some of the most up-regulated genes (Table 2) had at least one Hoxc4 consensus sequence; the up-regulation of these genes might therefore be explained by a direct reflection of the up-regulation of *Hoxc4*. It was observed that of the up-regulated genes having a Hoxc4 consensus sequence, none had a transcriptional regulatory function and therefore were not responsible for up-regulation of other genes. The finding of 'genetic disorder' pathway as one of the networks most affected reflects Crx's proven involvement in several retinal diseases (Furukawa *et al.* 1997, 1999).

In contrast to the absence of marked morphological changes in the pineal gland, the retina of $Crx^{-/-}$ mice is grossly affected as evident from the loss of photoreceptor outer segments (Furukawa *et al.* 1999; Rovsing *et al.* 2010). A large number of genes are also dysregulated in the retina of the $Crx^{-/-}$ mouse with major morphological changes to follow (Furukawa *et al.* 1999; Hsiau *et al.* 2007; Rovsing *et al.* 2010). Some of the genes which are down-regulated in the $Crx^{-/-}$ pineal gland are also down-regulated in the $Crx^{-/-}$ retina including *rhodopsin* (*Rho*) and *cone opsin* (*Opn1sw)* (Furukawa *et al.* 1999; Hsiau *et al.* 2007). A comparison of the most affected genes in both tissues (data from Hsiau *et al.* 2007) indicates that only a few are affected in both tissues. For example, *Cbln1* is down-regulated ~19-fold in the pineal gland but is not affected in retina, indicating that this gene is regulated by different mechanisms in these tissues. This is consistent with the explanation that Crx acts in concert with other transcription factors to control transcription (Nishida *et al.* 2003); apparently, the combina-

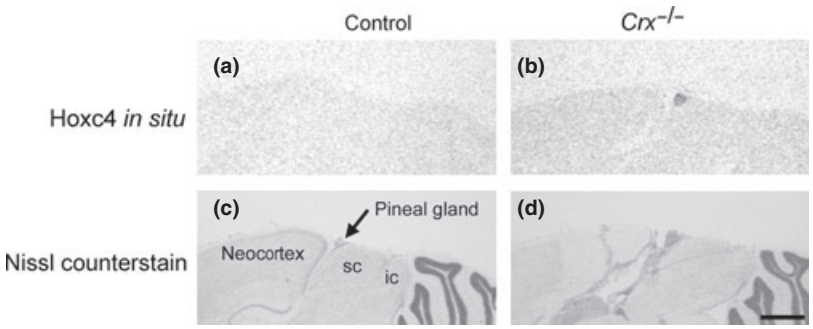

Fig. 4 Radiochemical *in situ* hybridization histochemical (ISH) detection of *Hoxc4* mRNA. Panels (a) and (c) are sagittal brain sections from a wild-type mouse and panels (b) and (d) are sagittal brain sections from a $Crx^{-/-}$ mouse. The upper panels are results from ISH and the lower panels are results of Nissl counterstaining of the sections used for ISH analysis. A positive ISH signal is only seen in the superficial pineal gland of the $Crx^{-/-}$ mouse (b) and not in that of the wild-type (a), in agreement with the results of microarray and qRT-PCR studies. A signal was not generated with a sense probe. Scale bar = 1 mm. For further details, see the Materials and methods section. sc, superior colliculus; ic, inferior colliculus.

tion of factors in the retina differs from that in the pineal gland.

Here, we found that the amplitude of the daily rhythm in *Aanat* transcripts is reduced 50% in the pineal gland of the $Crx^{-/-}$ mouse, in confirmation of previous observations (Furukawa *et al.* 1999). This indicates that a functional Crx is required for the normal pattern of expression of *Aanat*. It is not clear whether the absence of Crx appears to reduce Aanat expression by lowering the maximum level of expression or by shifting the timing of the peak in expression. The expression of Aanat is of special interest because it is thought that multiple Crx consensus sequences in the *Aanat* promoter play a conserved role in *Aanat* expression (Appelbaum and Gothilf 2006; Klein 2007). However, it is clear that Crx is not an absolute requirement for expression of *Aanat*. This does not, however, eliminate the role of the PCE sites. It is possible that the role of Crx might be redundantly played by the related transcription factor Otx2 which binds to the Otx variant of the PCE site. Otx2 is essential for pineal development (Nishida *et al.* 2003) and has a distinct postnatal role in the mouse retina (Koike *et al.* 2007). *Otx2* is expressed in the adult rodent pineal gland and retina in the presence and absence of Crx (Rath *et al.* 2006; Supplemental tables S3 and S5 in Hsiau *et al.* 2007). Otx2 and Crx have similar binding preferences and exhibit functional redundancy (Li *et al.* 1998; Bobola *et al.* 1999; Bernard *et al.* 2001; Dinet *et al.* 2006). Accordingly, it is reasonable to suspect that Otx2 substitutes for Crx in the pineal gland of the $Crx^{-/-}$ mouse in a redundant manner to control expression of *Aanat* and perhaps other genes. In the case of genes with consensus binding sequences for both, as is the case with *Aanat*, expression may only reflect the interaction of Otx2 with the Otx2 consensus binding sequence. Whereas it is also possible that up-regulation of *Otx1* or *Otx2* might compensate for decreased *Crx* expression, our microarray analysis failed to detect a change in expression of either *Otx1* or *Otx2*, thereby providing no reason to entertain such a compensatory explanation.

The reduction of the amplitude of *Aanat* expression in the $Crx^{-/-}$ mouse might also reflect reduction of expression of *Cbln1*, which is thought to modulate norepinephrine release (Albertin *et al.* 2000). Cbln1 might be normally released by pinealocytes into the extracellular space, where it could enhance norepinephrine release from sympathetic nerve endings. In addition, *Adcy3*, adenylate cyclase 3 is down-regulated 5.3-fold in the $Crx^{-/-}$ mouse. The product of this gene catalyzes the formation of cAMP (Linder 2006). *Cbln1* does not appear to be directly regulated by Crx because promoter analysis fails to reveal the presence of either a Crx or an Otx2 consensus sequence. Other possible scenarios include the impact of Crx elimination on visual function and control of Aanat levels and temporal organization of the transcription factor system controlling expression.

The results of this investigation also point to a role of Crx in circadian biology of the pineal gland, because there was no evidence in the $Crx^{-/-}$ pineal gland of a day/night pattern of expression of 43 genes seen in the wild-type animal. This may reflect temporal disorganization of the transcription factor system which controls gene expression, including Cry1 and Nr1d1/Rev-erbα. It is also possible that this is linked to the less robust nature of circadian locomotor activity seen in the $Crx^{-/-}$ mouse (Rovsing *et al.* 2010), which provides evidence for a role of Crx in circadian rhythms, separate from phenotype determination in the pineal gland and retina. Effects on circadian biology may reflect *Crx*-dependent changes in expression of a subset of the > 1000 genes that are dysregulated in the $Crx^{-/-}$ mouse pineal gland, some of which are involved in signal transduction (e.g. *Gng4*, *Rasgrf1* and *Rho*). It is also possible that Crx-dependent effects on the pineal gland contribute to the circadian amplitude of locomotor activity and temperature, both of which are reduced in the $Crx^{-/-}$ mouse

(Rovsing *et al.* 2010). It can be speculated that these effects are mediated by an unidentified pineal-dependent mechanism. It is unlikely that melatonin is involved because the capacity to synthesize melatonin is markedly reduced in the 129sv strain, which, as indicated above, is true of most laboratory mice (Goto *et al.* 1989).

As discussed above for *Aanat*, it is not clear if changes in differential day/night gene expression represent a true shift from constant non-day/night expression to day/night expression or if genes that appear to become expressed on a day/night basis in the $Crx^{-/-}$ mouse are also expressed with different phasing on a day/night basis in wild-type animals; and, that changes in the circadian system combined with the limitations imposed by a two point sampling experimental design resulted in the detection of these rhythms. Future studies involving more frequent time samplings should provide a better profiling of the influence of Crx on the temporal nature of gene expression in the pineal gland.

The adult pineal gland is not known to be photosensitive. The expression of phototransduction genes in this tissue appears to reflect the common origin of photoreceptors and pinealocytes (Klein 2007). If the transcripts are translated, the encoded proteins could function indirectly in G-protein coupled receptor signaling. For example, photoreceptors could form heterodimers with the adrenergic receptors which control pineal function. Formation of receptor heterodimers is known to modify function and specificity (Prinster *et al.* 2005).

Hoxc4 in the pineal gland

These studies have revealed a strong up-regulation of *Hoxc4* in the $Crx^{-/-}$ mouse pineal gland (Figs 3 and 4), which is not seen in the wild-type or $Crx^{-/-}$ retina by microarray (Hsiau *et al.* 2007) or qRT-PCR (data not shown). It seems reasonable to consider that the low level of *Hoxc4* expression in the wild-type pineal gland normally influences the transcriptome profile by not inducing expression of the genes which are up-regulated in the $Crx^{-/-}$ pineal gland. It is of interest to find that analysis of the *Hoxc4* co-citation network (Fig. 2) indicates that to a large degree, the genes linked to *Hoxc4* in other systems are not apparently linked to *Hoxc4* in the pineal gland. This suggests that Hoxc4 networks differ markedly on a tissue-to-tissue basis.

Summary

It appears that Crx plays a broad role in controlling the pineal transcriptome by weakly to mildly enhancing or suppressing expression of ~1200 genes. However, the change in the transcriptome is not associated with a remarkable change in cellular composition of the gland or in a dramatic loss of expression of pinealocyte marker genes. This indicates that Crx is not an absolute requirement for expression of the pinealocyte phenotype. Some of the changes observed in the absence of Crx expression could possibly be mediated in part by a marked increase in the downstream expression of the homeobox gene *Hoxc4*, which may normally play a less obvious role in shaping the pineal transcriptome.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1. Transcripts down-regulated 2- to 4-fold in the $Crx^{-/-}$ mouse pineal.

Table S2. Transcripts up-regulated 2- to 4-fold in the $Crx^{-/-}$ mouse pineal.

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